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Encapsulation and efflux of lactone and hydroxy acid forms of simvastatin in reverse-phase evaporation vesicles

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Summary

Simvastatin (SV) is the lactone prodrug of SVA, a hydroxymethylglutaryl-CoA reductase inhibitor, which in its active form of a hydroxy acid (SVA) lowers plasma cholesterol through inhibiting its endogenous synthesis. The present paper describes an attempt to encapsulate both drugs in reverse-phase evaporation (REV) vesicles. The experimental results can be summarized as follows: (i) both drugs (SV and SVA) are encapsulated efficiently into DPPC liposomes with high yield; (ii) in all preparations tested, the more hydrophilic SVA is encapsulated to a considerably greater extent than SV, reaching approx. 60% in the case of DPPC liposomes; (iii) the presence of cholesterol in the vesicle wall markeldy reduces this capacity; (iv) it is possible to control the release of drug from the liposomes by modifying the lipid composition of the vesicles. The procedure of encapsulation into liposomes in principle should permit the direct administration of SVA, thereby reducing the toxicity associated with high doses of SV.

Introduction

Simvastatin (SV) is a very potent and specific inhibitor of the microsomal enzyme 3-hydroxy-3-methylglutaryl-CoA reductase [HMG-CoA reductase; mevalonate : $NADP^+$ oxidoreductase (CoA acylating) EC 1.1.1.34]. This agent has been shown to be a highly effective cholesterol lowering drug in various animal species including humans, modulating the endogenous synthesis of this sterol (Alberts, 1988; Germershausen et al., 1989; Tikkanen, 1989).

Simvastatin as a lactome is the prodrug form and is transformed in vivo to the biologically active β-hydroxy acid form, SVA (Fig. 1). The lactone inactive form has been observed to be subject to a greater degree of hepatic extraction from the circulation than is the β-hydroxyl derivative (Gerson et al., 1989) and, therefore, is

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Abbreviations: CHO, cholesterol; DPPA, dipalmitoyl-1-α-phosphatidic acid; DPPC, dipalmitoyl-1-α-phosphatidylcholine; SA, stearylamine, SV, simvastatin lactone form; SVA, simvastatin β-hydroxy acid form; REV, reverse-phase evaporation vesicles; TLC, thin-layer chromatography.

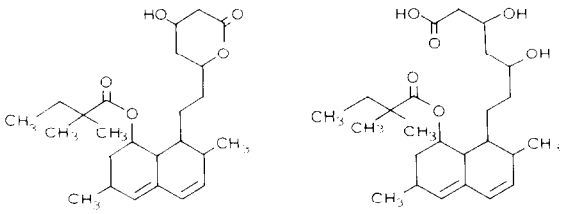


Fig. 1. Structures of SV (lactone form) and SVA (hydroxy acid form).

usually administered in clinical use. The hydrolysis of simvastatin in its active carboxyl form, which can be obtained chemically in a strong alkaline medium (Takano et al., 1990), is achieved in vivo by the action of liver esterases (Alberts, 1988).

The aim of the present investigation was to study the entrapment of simvastatin or its β-hydroxyl derivative into liposomes which might constitute a delivery system for this drug.

In particular, both drug forms were entrapped in reverse-phase evaporation phosphatidylcholine vesicles (REV liposomes) and by means of a simple spectrophotometric method (Morelli, 1988; Di Giulio et al., 1989, 1991) the effect of liposomal composition on the entrapment efficiency and on the release of both drugs was investigated.

Materials and Methods

Chemicals

Simvastatin (MK-733) was supplied by Merck Sharp and Dohme Italia S.p.A.; cholesterol (CHO), dipalmitoyl-1-α-phosphatidylcholine (DPPC) and dipalmitoyl-1-α-phosphatidic acid sodium salt (DPPA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); ethyl acetate and hexane were from Fluka (Fluka Chemie, Buchs, Switzerland); thin-layer silica gel 60A plates fluorescent at 254 nm were obtained from Whatman Int. Ltd (Maistone, U.K.); chloroform, methanol and other chemicals were reagent grade from Merck (Darmstadt, Germany).

Equipment

Equipment consisted of a Lab-line Ultratip sonicator (Lab-line Instr., Melrose Park, IL, U.S.A.), a rotary evaporator (Büchi HB-140, Büchi Instr., Flawil, Switzerland), a UV GL-58 lamp (UV Products Inc, San Gabriel, CA, U.S.A.), a Centrikon T-124 ultracentrifuge and a Uvikon 860 double-beam UV-Vis spectrophotometer with a 1 cm quartz cell (Konstron Instr., Milano, Italy). Suitable settings for the measurements were: bandwidth, 2 nm; response time, 1 s; scan speed, 100 nm/min. The recorder scale expansion was

TABLE 1

SV and SVA encapsulation and efflux for various types of reverse-phase evaporation liposomes

Lipid composition (molar ratio)	Drug used for entrapment	Intraliposomal drug concentration (μM) [a]	Encapsulation (%) [a]	Drug efflux after 48 h (μM) [a]
DPPC	SV	126.0 ± 5.3	31.5 ± 1.3	23.5 ± 2.7
	SVA	269.0 ± 27.1	67.2 ± 6.8	33.3 ± 6.4
DPPC:CHO	SV	7.3 ± 1.8	1.8 ± 0.4	N.D.
(7:3)	SVA	53.5 ± 3.9	13.3 ± 0.9	33.7 ± 1.2
DPPC:CHO	SV	4.6 ± 1.2	1.2 ± 0.3	N.D.
(5:5)	SVA	34.6 ± 2.3	8.6 ± 0.6	16.5 ± 0.5
DPPC:SA	SV	96.4 ± 1.4	24.1 ± 0.5	41.6 ± 2.3
(8:2)	SVA	110.0 ± 7.1	27.6 ± 1.8	46.6 ± 4.7
DPPC:SA:CHO	SV	70.5 ± 7.8	17.6 ± 1.9	30.8 ± 5.3
(7:2:1)	SVA	118.0 ± 28.3	29.5 ± 7.1	49.5 ± 16.8

[a] The initial SV and SVA concentrations used in liposome preparations were 400 μM and are referred to as 100% for the corresponding samples of loaded vesicles. Each value is expressed as the mean ± S.D. of three independent experiments.

optimized to facilitate readings on the recorder tracing.

Preparation of SV- or SVA-loaded liposomes

A sample of simvastatin (5 mg) was dissolved in 60 ml of 1 : 1 (v/v) methanol-0.1 M phosphate buffer (pH 7.2). Methanol was removed by rotary evaporation under reduced pressure at 30 °C to give a final concentration of 4×10^{-4} M. Sometimes, after removal of the organic solvent, SV partially precipitated yielding a lower final concentration. In parallel, 5 mg of SV were hydrolysed in the hydroxy acid derivative, SVA, by reaction with 60 ml 1 : 1 (v/v) methanol-0.1 KOH for 1 min at room temperature. After neutralizing the solution with concentrated HCl, the methanol was removed under reduced pressure. The SV and SVA solutions were used to load vesicles, according to the method of Szoka and Papahadjopoulos (1978). In particular, in each preparation 50 μmol of different lipidic cocktails were used as reported in Table 1.

Control of purity SV and SVA

From 1 ml of 4×10^{-4} M solutions, SV and SVA were extracted with 3 ml of chloroform and, after concentration by partial evaporation of the solvent, layered on fluorescent silica gel 60A plates and eluted with ethyl acetate-hexane 7 : 3 (v/v). The spots were revealed using a UV GL-58 lamp at 254 nm.

Derivative measurements and release experiments

The derivative spectrophotometric measurements used were the so-called 'peak-through amplitude' between a maximum and a minimum, as reported by Morelli (1988). This method allows one to determine the extent of liposome entrapment avoiding the noise due to sample turbidity (Di Giulio et al., 1989, 1991). In this case, the best correlation between the peak-through amplitude and simvastatin concentration, for either the lactone or hydroxy acid form, was +240 nm for the maximum and −245 nm for the minimum. Monitoring these peaks, the following regression curve between third derivative values (y) and drug concentrations (x) was used for calculations:

$$y = 2.73 \times 10^{-4}\, x + 0.03 \quad (r = 0.999)$$

As far as the release experiments are concerned, 4 ml of loaded-liposomes suspension, containing 50 μmol of total lipids, were kept at room temperature and at fixed intervals (0, 2, 5, 24, 48 h), the suspension was carefully vortexed and 500 μl aliquots were withdrawn. These aliquots were centrifuged at $30\,000 \times g$ for 15 min at 10°C and the pellets were resuspended in fresh buffer up to the original volume. 100 μl of these suspensions were used to determine the residual entrapped drug, calculated as a percentage with respect to 100 μl of the initial loaded-liposome suspension, which was taken as 100%. In one series of experiments concerning the release of stearylamine containing DPPC loaded-liposomes, the effect of the polyanion heparin was also studied. To 1 ml of vesicle suspension containing 12.5 μmol of total lipids kept at room temperature for 48 h, 50 μmol of heparin were added under continous vortexing. The sample was placed at room temperature for a further 4 h and, then, read for the residual trapped drug. Each measurement was made in triplicate.

Results and Discussion

Starting from the literature data on the pharmacological action of simvastatin, for either the prodrug form of lactone (SV) or its hydroxy acid derivative (SVA Germershausen et al., 1989; Gerson et al., 1989), we undertook a study on the encapsulation and release of both drugs from vesicles of different composition and under various experimental conditions. Firstly, the lactone in the hydroxy acid form was hydrolyzed by treatment with a strong alkali using methanol-0.5 M KOH 1 : 1, v/v). This reaction yields the active drug (Takano et al., 1990) which is much more hydrophilic. After elution with ethyl acetate-hexane mixture, TLC analysis showed a single spot corresponding to SV with a rough R_f of 0.5 (Fig. 2), whereas the hydroxy acid form, which was extracted from the corresponding solution in the same way, is scarcely eluted with an R_f of about 0.12, indicating the complete transformation of the drug. In this context, it is interesting to note that the hydrolysis of SV into SVA also

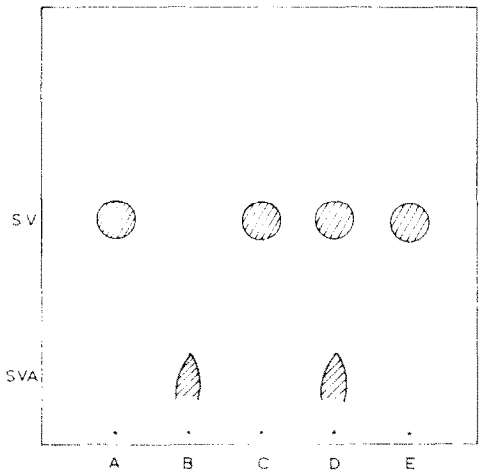


Fig. 2. Analysis of simvastatin, lactone or hydroxy acid form, by TLC. Sample (A) SV dissolved in chloroform; (B) chloroform extract of SV after methanol-0.1 M KOH (1:1, v/v) treatment; (C) SV in methanol-0.1 M (1:1, v/v) phosphate buffer (pH 7.2); (D) as in (C) after 24 h; (E) SV in methanol.

takes place at neutral pH in methanol-phosphate buffer (1:1, v/v), although to a lesser extent, resulting in 50% transformation after about 24 h.

As far as the entrapment and release experiments are concerned, we utilized liposomes prepared by the reverse-phase evaporation method according to Szoka and Papahadjopoulos (1978), since they have a high capture volume and are mostly unilamellar. To estimate the extent of drug entrapment, a method based on derivative spectrophotometry was employed (Di Giulio et al., 1989, 1991), which allows one to calculate directly the SV or SVA content in the vesicles, without disruption of their structure or use of radiolabeled tracers. Fig. 3 demonstrates zero- and third-derivative spectra of both forms of the drug, in solution or entrapped in liposomes.

Using this approach, we calculated the liposomal entrapment efficiency as a function of the

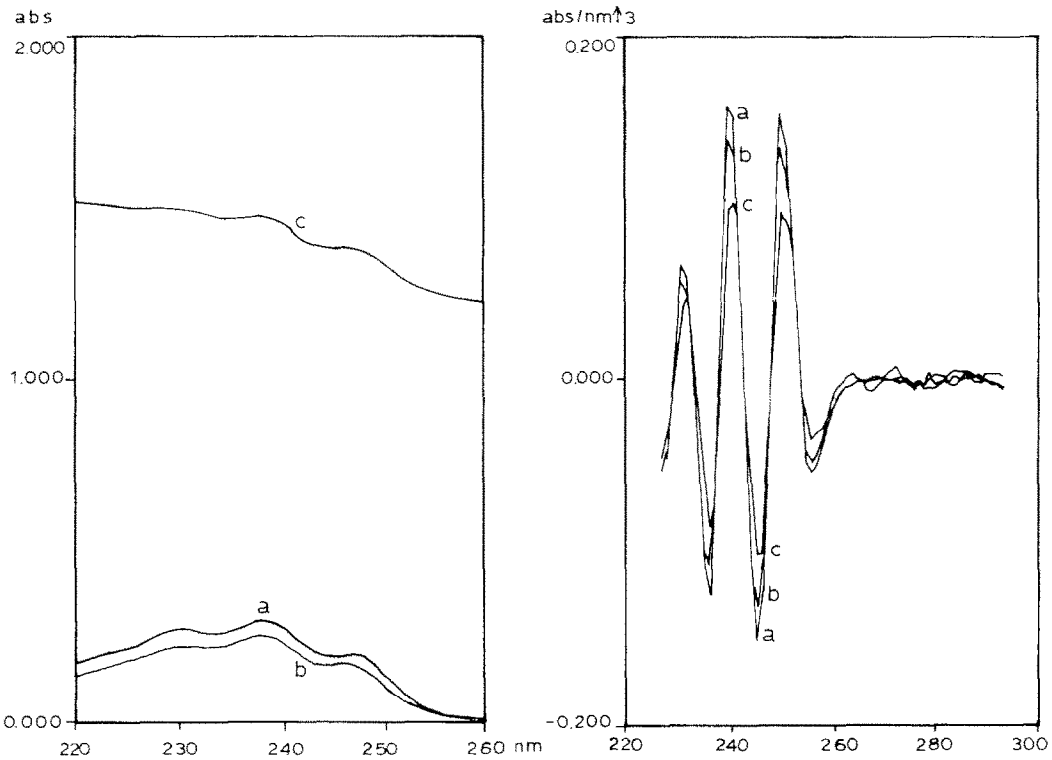
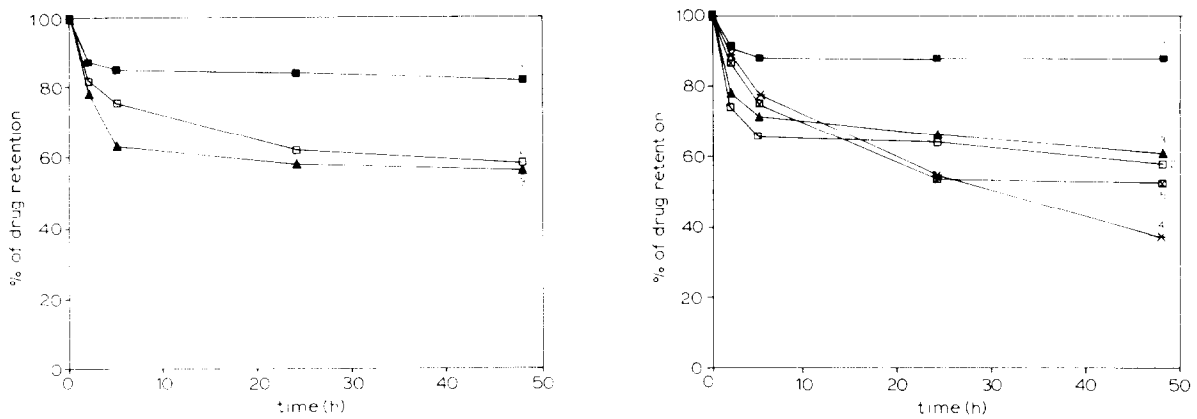


Fig. 3. Zero- (left) and third-order (right) derivative spectra of SV and SVA preparations. (a) Lactone form of simvastatin (SV), in phosphate buffer; (b) hydroxy acid form of simvastatin (SVA), in phosphate buffer; (c) SV-loaded DPPC vesicles suspension in phosphate buffer. For experimental details see Materials and Methods.

lipidic cocktail, as reported in Table 1. It can be noted that in all the liposome preparations the more hydrophilic SVA was entrapped to a greater extent than SV, reaching encapsulation percentage for SVA and SV of 67 and 31%, respectively. This value fell dramatically when cholesterol was added to the lipidic shell, however, this effect was more pronounced for SV: in fact, in liposomes composed of DPPC:CHO (1:1, molar ratio) the lactone entrapped was around 1%. Furthermore, the presence of positive charges on the liposomal surface, i.e., liposomes containing stearylamine, also lowered the entrapment efficiency. This observation appears to indicate that the interaction between liposomal surface charge and the negative charge of the acidic group in SVA does not play a determinant role in the process of entrapment; in other words, one could argue that the absorption of the drug on the surface and/or the solubilization in the lipidic bilayer was not significant, whereas the solubility of SV and SVA in the inner aqueous volume of the vesicle could account mostly for the encapsulation process.

The efflux of drugs from vesicles was followed during a time lag of 48 h after loading liposomes. This phenomenon also appears to be clearly correlated to the lipid composition used in the liposome preparation (Fig. 4). For both SV forms, DPPC liposomes leaked up to 20% of the carried drug during the measurement time of the experiment, whereas cationic vesicles, which had lower entrapment capacity, showed more pronounced release in bulk solution for both drug forms, around 40% of their content. The presence of cholesterol strongly facilitates the efflux of SVA; due to the low entrapment capacity of SV in the latter type of liposomes, it was impossible to study the effect of cholesterol on the release of SV.

As reported previously polyanions cause aggregation of cationic vesicles (Felgner et al., 1987; Felgner and Ringold, 1989). In this context the addition of a polyanion such as heparin to cationic 48 h old liposomes with a residual SVA entrapment of 57.6 ± 4.7% led to a coalescence of vesicles being observed with a sensitive increase in



Sample no.	Molar ratio of DPPC:CHO:SA
1	10:0:0
2	8:0:2
3	7:1:2
4	7:3:0
5	5:5:0

Fig. 4. Time course of SV (left) and SVA (right) efflux from various REV liposome preparations.

drug efflux after a further 4 h, which lowers the content of entrapped drug to a value of only $23.5 \pm 3.5\%$. These data may be explained on consideration of the fact that the coalescence of vesicles leads to the rupture of membranes which causes the release into the bulk solution of the hydrophilic SVA.

In principle, this new procedure may eventually permit one to regulate, by modifying the composition of the liposomal shell, the target organs of drug and allow at the same time the controlled release of the therapeutical agent.

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